RAPID QUANTIFICATION OF DIPICOLINIC ACID FROM SPORES TREATED BY PRESSURE-ASSISTED THERMAL PROCESSING USING INFRARED SPECTROSCOPY

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ABSTRACT

Endopores (or spores), which are produced by certain bacteria, are capable of surviving food processing and causing food spoilage and foodborne illness. Dipicolinic acid (DPA), which constitutes about 10% of the spores' dry weight, plays a major role on wet heat resistance of spores. Current methods like chromatography and fluorometry that are used to quantify DPA are time-consuming, expensive and use hazardous solvents. Rapid methods to quantify the DPA levels in spores would enable simple and cost-effective approaches to study spore inactivation during thermal and non-thermal food processing. FTIR spectroscopic analysis was used to build a regression model that could be used for rapid prediction of DPA content released from treated *Bacillus* spores. *Bacillus amyloliquefaciens* FAD82 cultures were treated by pressure-assisted thermal processing with sampling at different time intervals. Aliquots (1mL) of the processed samples were centrifuged and the supernatant was analyzed using a fluorometer (reference method) to determine the released DPA concentration. Pellets were dried on a hydrophobic grid membrane filter and scanned using FTIR spectrometer. The collected spectra were correlated with the DPA concentrations obtained from the fluorometer to develop regression model based on partial least square regression analysis. The regression model was able to predict the DPA concentrations with a standard error of 6 μM. This method proved to be simple, rapid and accurate, without the need for expensive and hazardous chemicals.

INTRODUCTION

Bacterial endospores (spores) are dormant structures produced by vegetative bacterial cells belonging to genus *Bacillus, Alicyclobacillus* or *Clostridium*. Spores generally demonstrate higher resistance than vegetative cells to processing conditions like heat, radiation and chemical disinfectants [1]. Inactivation of bacterial spores is crucial for sterilization of food and pharmaceutical product. Thermal sterilization of foods inactivates bacterial spores of public health significance, but the typical high temperatures and long heating times adversely affect the nutritional and organoleptic characteristics of most foods. Less detrimental alternative processing technologies such as pressure-assisted thermal processing (PATP), ohmic heating and microwave processing are desirable. However, the microbiological safety of these alternative processing technologies must be ensured for the production of safe food products.

Though information about the factors that contribute to the high heat resistance of spores are known, the exact nature of spore inactivation by these factors remain obscure. These factors include the protection of spore DNA by small-acid soluble proteins, the accumulation of divalent cations, such as $Ca2^{+}$ and $Mn2^{+}$, and the dehydration of the spore core [2]. In addition, there is a role in heat resistance for dipicolinic acid (pyridine-2,6-dicarboxylic acid; DPA), to which the divalent cations are chelated in the core of the spore. Spores accumulate large amount (5 to 15% of dry weight) of the spore-specific molecule DPA that is located in the spore's central region or core as a 1:1 chelate with divalent cations, predominantly Ca^{2+}. Recent work has indicated that Ca-DPA plays important roles in both spore resistance and stability by lowering the core water content and protecting spore DNA against a variety of damaging agents [3]. This process can elevate spore resistance to wet heat by protecting core proteins from inactivation or denaturation. Ca-DPA levels in dormant *Bacillus* spores can vary with the species, strain, and sporulation

conditions and the spore's DPA content can markedly influence its resistance properties. Variations in Ca-DPA content might be correlated with heterogeneity in resistance properties of spores in a population. Thus, DPA is regarded as a major biomarker for detection of bacterial spores and determination of spore inactivation [1, 3-5].

Several researches have been performed to investigate the effect of DPA content on the resistance of spores to processing conditions [6-9]. All these studies employ laborious and time-consuming techniques like traditional plating techniques, extraction methods, chromatographic techniques and fluorescence. The chemical methods also employ hazardous solvents as an integral part of the technique. The analysis time is further prolonged by the longer incubation periods required for spores. Fourier-transform infrared (FTIR) spectroscopy has been proposed as an alternative analytical technique of microorganisms by many researchers, because of its inherent advantages such as simplicity, rapidity, and high sensitivity and throughput [10, 11]. FTIR spectroscopy is a rapid technique for monitoring chemical constituents in complex matrices, including DPA, during spore inactivation [12]. Cellular constituents like polysaccharides, proteins, nucleic acid and other molecules provide specific patterns in the spectra that are unique for each microorganism. Extensive research has been conducted on characterizing microorganisms using FTIR spectroscopy and the technique was recently extended to study the composition of spores and to quantify spores [13, 14]. FTIR spectroscopy can provide real-time results and unlike conventional methods, it gives information on biochemical and structural composition along with DPA content. **The objective** of this study was to develop a simple, reagent-less and rapid method to quantify the DPA released from spores during pressure-assisted thermal processing by FTIR spectroscopy.

MATERIALS AND METHODS

Sporulation conditions:

Bacillus amyloliquefaciens TMW 2.479 FAD82 spores were used in the study due to their high pressure-thermal resistance [15-17]. Cultures were grown in trypticase soy broth supplemented with 0.6% yeast extract (TSBYE) with aerobic incubation at 32°C for 24 h. After the second transfer in TSBYE, the cultures were used for spore production. Two batches of spores were produced using two different media. The first batch was prepared by spread-plating the 100 µl portions of B. amyloliquefaciens culture on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) and 10 ppm $MnSO_4 \cdot H_2O$. The second batch was grown on nutrient agar supplemented with 0.6% yeast extract (NAYE) and 10 ppm $MnSO_4 \cdot H_2O$. The inoculated plates on TSAYE were aerobically incubated at 32°C for 10-14 days, whereas those of NAYE were incubated for 3-5 days to obtain 95% sporulated population. The sporulation was verified by using a phase-contrast microscopy. The surface of inoculated plates was flooded with 10 ml of cold sterile deionized water and the spores were scraped with disposable plastic spreaders. The spore suspension was washed five times by differential centrifugation that ranged from 2000 to 8000 rpm for 20 min each at 4°C. Spore pellets were re-suspended in sterile deionized water to obtain ~10^8 spores/ml for each production. The suspension was sonicated for 10 min following by heat treatment at 80°C for 10 min to destroy any remaining vegetative cells. The spore suspension was stored in a refrigerator at 4°C until used.

Processing:

The pressure treatment experiments were carried out using PT1 (Avure Technologies, Kent, WA). The lab scale equipment has approximately 30 s pressure come-up time. Spores (10^8 spores/ml) suspensions were subjected to PATP (600 MPa and 105°C), HPP (600 MPa and

35°C) and pressure pulsing (600 MPa and 105°C with pulsing) for selected holding times. Spores treated with pressure pulsing was used only for prediction. Samples (1 ml), collected at regular time intervals, were centrifuged (13000 rpm and 4°C for 4.5 min) and the supernatant was used for fluorescence assay and the pellet for FT-IR spectroscopy.

Fluorimetric assay:

Sample supernatants were analyzed using a fluorometer to determine the DPA concentration. The fluorescence of DPA in spores is based on the emission of terbium (Tb3+) ion upon binding with DPA forming $(Tb\text{-}DPA)^{+}$ complex. DPA solutions ranging from 1 to 20 µM were prepared for the standard curve. Terbium reagent was prepared using 100 µM terbium chloride hexahydrate and 20 mM Tris buffer at pH 7.5 [18]. The DPA sample and terbium reagent (1:1) were mixed and the fluorescence was monitored using Cary Eclipse Fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA) at 225 nm excitation and 545 nm emission settings. The released DPA of pressure treated samples were obtained from the standard curve.

FTIR spectroscopic analysis:

Bacterial spore pellets (2µL) were applied onto hydrophobic-grid membranes (HGM). HGM uses a grid pattern, printed in hydrophobic material to separate colonies from one another allowing for orderly arrays of bacterial samples [Figure 2 insert]. The isolated bacterial spores were measured using FTIR microspectroscopy (Varian 3500GX, Palo Alto, CA) and the resulting spectra were analyzed using multivariate analysis software.

Multivariate analysis:

The collected spectra were imported into Pirouette® (Infometrix Inc., Bothel, WA) software, mean-centered, transformed into their 2^{nd} derivative and normalized prior to analysis.

The relationship between specific fingerprint spectral information and DPA concentration obtained from the fluorimetric assay (reference method) was determined using partial least squares regression (PLSR). PLSR models with cross-validation were developed to predict the DPA concentrations of pressure treated *B. amyloliquefaciens* FAD82 spores. The predictability of the model was tested using five independent unknown samples.

RESULTS AND DISCUSSION

Spores of *B. amyloliquefaciens* FAD82, grown in TSAYE and NAYE, were subjected to HPP and PATP for different time intervals. The amount of released DPA during treatment was determined by fluorescence spectroscopy as reference method. The data exhibited excellent fit with a coefficient of determination (R^2) of >0.99 (data not shown).

The amounts of DPA released from HPP and PATP-treated spore samples, determined using the fluorescence standard curve, are summarized in Table 1. A significantly greater amount of DPA release was observed during PATP than HPP ($p<0.05$). Furthermore, spores grown in NAYE exhibited greater DPA release than spores grown in TSAYE. Spores grown in NAYE have been reported to have a higher D-value (the time required to reduce the population by 90% at a particular temperature) than those grown on TSAYE [19]. Therefore a greater DPA release observed in *B. amyloliquefaciens* spores grown on NAYE could be interpreted as higher initial concentration of DPA in spores grown on NAYE than TSAYE. The higher concentration of DPA may in turn be responsible for the greater resistance of spores grown on NAYE.

The feasibility of using infrared spectroscopy as a rapid and simple tool for quantifying DPA release was evaluated using pressure-treated samples. Drying spore pellets on hydrophobic grid membrane resulted in a well contained uniform spot of sample that allowed for the

collection of high-quality spectra with distinct and consistent spectral features. The raw spectra were transformed into their second derivatives for analyses to remove the baseline shifts, improve the peak resolution, and reduce the variability between replicates [20]. Raw spectra of *Bacillus amyloliquefaciens* FAD82 is shown in Figure 1. FTIR spectra reflect the total biochemical composition of the bacterial spore, with bands due to major cellular constituents such as water, lipids, polysaccharides, acids, etc. The region from 4000 to 3100 cm^{-1} consists of absorbance from O-H and N-H stretching vibrations of hydroxyl groups and Amide A of proteins, respectively. Protein bands also appear in the regions 1700 – 1550 cm^{-1} (amide I and amide II) and 1310 – 1250 cm^{-1} (amide III). The spectral range 1250 – 800 cm^{-1} consists of signals from phosphodiesters and carbohydrates. The region between 1800 and 1200 cm^{-1} has been reported to contain almost all signals of interest in studying spore inactivation during PATP and thermal processing [14].

The transformed spectra of treated samples were correlated with their corresponding DPA release value determined using fluorometry as a reference method to develop a PLSR model Figure 2. The coefficient of correlation (r-value) between IR predicted DPA release and reference DPA release (fluorimetric assay) was >0.98. The estimated error expected in predicting unknown samples (SECV; standard error of cross-validation) was 6.26 µM. Over 90% of the variation could be explained within as few as 3 principal component factors. Figure 3 shows the prominent wavenumbers (Factor 1) that had high influence on the regression model. Almost all of the significant absorption bands were of DPA and its compounds: 1616, 1570, 1439, 1378 and 1281 cm^{-1}. A description of each of these DPA bands and their type of absorption is presented in Table 2. This clearly shows the potential of this technique to specifically monitor DPA bands and extract useful information related to biochemical and structural changes taking place during

processing. Some minor changed in protein related bands (1412 cm^{-1}) were also observed during treatment.

B. Amyloliquefaciens FAD82 spores grown on TSAYE and treated with pressure pulsing was used as a prediction set to validate the PLSR model. Table 3 summarizes the DPA concentrations measured by the fluorimetric assay and predicted by FTIR spectroscopy. The FTIR predictions for independent samples were close to the expected standard error (6.26 μM) of the DPA concentrations obtained from the reference method. It is important to note that these variations in FTIR predictions also included variations from the reference fluorimetric method. The spectra collection time per sample was 30 sec. This model indicates that the combination of FT-IR spectroscopy combined with multivariate analysis can be used to develop reliable models for the rapid quantication of DPA release from spores regardless of the processing method or growth media.

CONCLUSIONS

The DPA release observed in *B. amyloquefaciens* FAD82 spores grown in two different media during PATP and HPP were successfully correlated with FTIR spectra. The PLSR model effectively predicted the DPA concentrations of spores treated by pressure pulsing. This technique shows great promise for quantification of DPA release regardless of the sporulation media and processing conditions, and offers advantages such as speed, accuracy, and cost-effectiveness without the need for reagents or extensive labor. It can significantly simplify quantification of DPA and provide an effective tool to predict the resistance of spores by aiding in the understanding of spore inactivation mechanism and related biochemical changes.

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Table 1. Amount of DPA released (uM ± standard deviation) by *Bacillus amyloliquefaciens* FAD82 spores grown on TSAYE and NAYE during HPP and PATP treatment

Released DPA (μM)					
HPP			PATP		
Time	**TSAYE**	**NAYE**	**Time**	**TSAYE**	**NAYE**
Control	4.95 ± 0.24	3.99 ± 0.92	Control	4.44 ± 0.21	4.05 ± 0.26
0 min	5.36 ± 0.34	3.34 ± 0.45	0 min	12.59 ± 0.44	49.41 ± 0.94
5 min	6.75 ± 0.23	4.59 ± 1.03	0.5 min	39.66 ± 2.19	209.65 ± 9.92
8 min	6.79 ± 0.31	4.26 ± 0.90	1 min	64.53 ± 1.24	243.81 ± 3.24
30 min	8.04 ± 0.46	12.00 ± 0.15	1.5 min	76.48 ± 2.01	343.37 ± 8.05
70 min	9.44 ± 0.41	38.82 ± 7.16	2 min	90.18 ± 1.16	353.06 ± 25.93
			3 min	88.58 ± 2.13	367.75 ± 25.53
			5 min	99.47 ± 2.35	375.10 ± 14.90
			8 min	116.75 ± 3.01	399.25 ± 14.43

Table 2. Absorption bands of important wavenumbers in the FTIR spectra of pressure-treated *Bacillus amyloliquefaciens* FAD82 spores

Wavenumbers (cm^{-1})	Functional groups[a]
~1281	amide III bands of proteins / DPA band
~1377	stretching bands of COO- group of Ca-DPA chelate
~1412	C-O-H in-plane bending of proteins
~1439	DPA pyridine ring vibration
~1570	C-N vibrations of the DPA ring
~1616	stretching bands of COO- group of Ca-DPA chelate

[a] summarized from references 5, 11, 12, 14 and 20.

Table 3. FTIR-predicted DPA concentrations (uM) released from *Bacillus amyloliquefaciens* FAD82 spores treated by pressure pulsing

Released DPA (μM)	
Measured by fluorometry	**FTIR predicted**
8.00	5.60
108.82	98.79
112.97	100.22
117.71	104.45
123.48	109.69

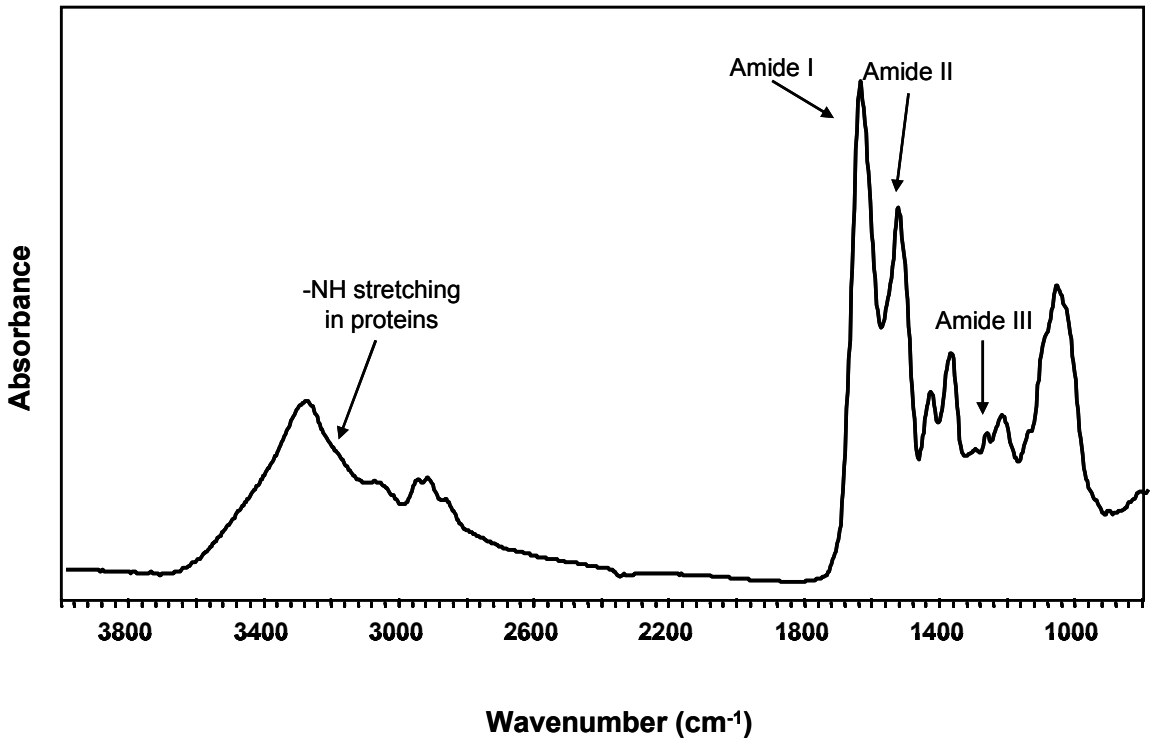

Figure 1. Fourier transform mid-infrared spectra of *Bacillus amyloliquefaciens* FAD82 spore pellet, prepared on hydrophobic grid membrane and scaned in a FTIR microspectrometer

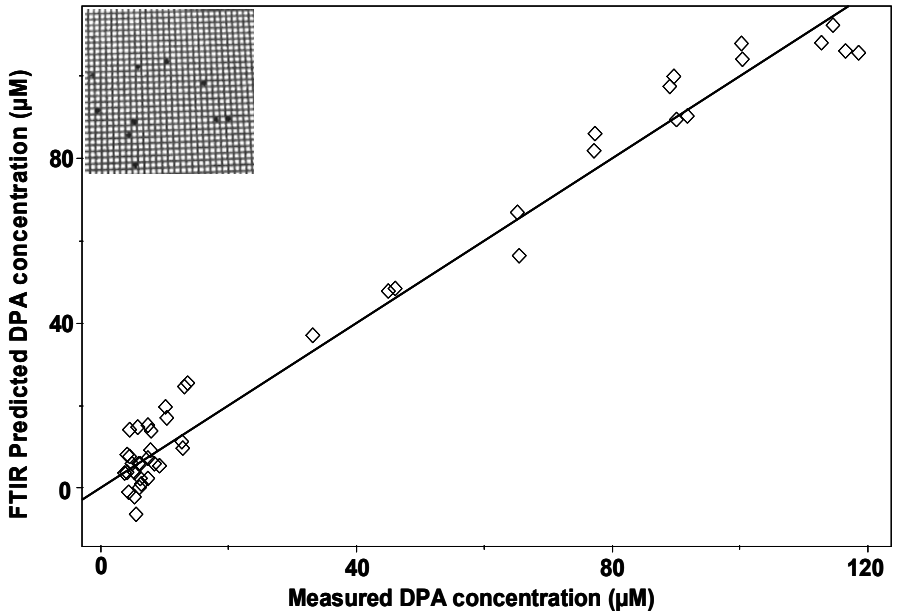

Figure 2. Cross-validated (leave-one-out) Partial Least Squares regression plot for estimation of DPA concentration. The **insert** shows a typical hydrophobic grid membrane with applied bacterial spore samples

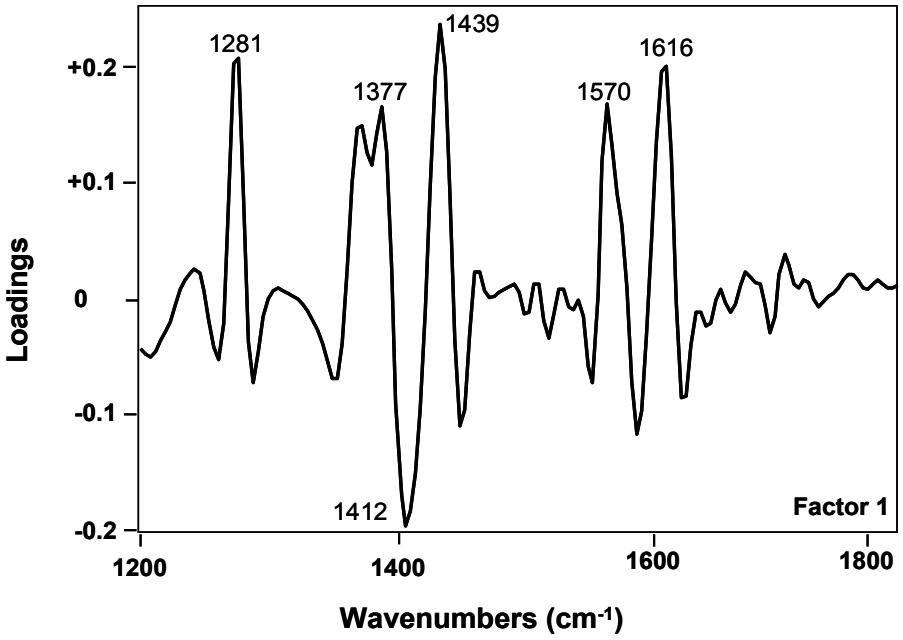

Figure 3. Loading spectra plot for the first latent variable of the training set. Important wavenumbers that loaded significantly (both high and low) are marked. A greater the loading the greater is the importance of the wavenumber in the PLSR model.